

Stem cells having increased sensitivity to a chemoattractant and methods of generating and using same.

FIELD OF THE INVENTION

5 The present invention relates to stem cells which exhibit increased sensitivity to a chemoattractant and, more particularly, to methods of generating and using them such as in clinical applications involving stem cell transplantation.

BACKGROUND OF THE INVENTION

10 Medical treatments of disorders caused by abnormal organ function typically employ pharmaceutical agents designed for either compensating for such abnormal organ function or treating the dysfunctional organ tissue. However, in some cases, pharmaceutical therapy cannot be instated since organ function is oftentimes complex and/or not completely understood.

15 In such cases, the only viable alternative is surgical replacement of the non-functional organ, which is now widely used for treatment of liver and kidney failure, both acute and chronic, as well as for cancer and certain inborn abnormalities. However, the need for donor organs far exceeds the supply. Organ shortage has resulted in new surgical techniques, such as splitting adult organs for transplant. Despite fairly good results, such techniques still suffer from a lack of donor tissue.

20 The lack of viable donor tissue has led to the emergence of stem cell replacement therapy, which relies on stem cell plasticity i.e., the ability to give rise to cell types in a new location that are not normally present in the organ in which the stem cells are located.

25 Stem cells are generally classified according to their origin, essentially adult, embryonic or neonatal origin. Embryonic stem cells deriving from the inner cell mass of the blastocyst are pluripotent, being capable of giving rise to cells found in all three germ layers. Despite long held belief adult stem cells are not as lineage restricted as previously thought. In particular, haematopoietic and neural stem cells appear to be the most versatile at cutting across lineage boundaries. For example,
30 recent reports suggest that hematopoietic stem cells (HSCs) of human origin have a hepatic potential. Studies of liver or bone marrow transplantation from sex mismatched donors, identified bone marrow-derived hepatocytes in recipients [Alison (2000) Nature 406:257; Theise (2000) Hepatology 32:11-16; Korbiling (2002) N Engl J

Med 346:738-746.]. Murine and rat HSCs were also found to migrate to irradiated or injured adult livers, and to differentiate into hepatic cells [Petersen (1999) Science 284:1168-1170; Theise (2000) Hepatology 31:235-240; Lagasse (2000) Nat Med 6:1229-1234]. Furthermore, single murine hematopoietic stem cell transplantation has
5 resulted in detection of HSC-derived cells in the liver of irradiated recipients with a low percentage of transplanted cells exhibiting immunohistochemical and morphologic properties of hepatic epithelial cells [Krause (2001) Cell 105:369-377].

The mechanisms that guide circulating hematopoietic stem cells are clinically significant because the success of stem cell transplantation depends on efficient
10 targeting (also referred to as homing) of grafted cells to the recipient target tissue [Mazo and von Adrian (1999) Journal of leukocyte Biology 66,25-32]. It is due to this homing of transplanted cells that bone marrow transplantations do not require invasive surgery, as in the case with the transplantation of any other organ, but rather can be effected by simple intravenous infusion.

15 Homing of HSCs can be defined as the set of molecular interactions that allows circulating HSCs to recognize, adhere to, and migrate across bone marrow endothelial cells resulting in the accumulation of HSCs in the unique hematopoiesis-promoting microenvironment of the bone marrow. Homing of progenitor cells can be conceived as a multi-step phenomenon [Voermans (2001) J. Hematother. Stem Cell Res. 10:725-
20 738, Lapidot (2002) Leukemia 16:1992-2003]. HSCs arriving to the bone marrow must first interact with the luminal surface of the bone marrow endothelium. This interaction must occur within seconds after the HSCs have entered the bone marrow microvasculature and provide sufficient mechanical strength to permit the adherent cell to withstand the shear force exerted by the flowing blood. Adherent HSCs must
25 then pass through the endothelial layer to enter the hematopoietic compartment. After extravasation, HSCs encounter specialized stromal cells whose juxtaposition supports maintenance of the immature pool by self-renewal process in addition to lineage-specific HSCs differentiation, proliferation and maturation, a process that involves stroma-derived cytokines and other growth signals.

30 Only a limited number of factors involved in stem cells homing are known to date; these include, the ligand for c-kit, stem cell factor, which has been shown to play a central role in adherence of HSCs to the stroma; and integrin interactions (e.g., β 1-Intergrins), which were shown to be crucial to the migration of HSCs to the foetal

liver [Zanjani (1999) Blood 94:2515-2522]. One important molecular interaction which is considered central to HSC homing is that of chemokine stromal derived factor (SDF-1) and its cognate receptor, CXCR4.

SDF-1 is the only known powerful chemoattractant of hematopoietic stem cells of both human [Aiuti (1997) J. Exp. Med. 185:111-120] and murine origin [Wright (2002) J. Exp. Med. 195:1145-1154] known to date. SDF-1 is widely expressed in many tissues during development [McGrath (1999) Dev. Biol. 213:442-456] and adulthood [Nagasawa (1994) Proc Natl Acad Sci U S A 91:2305-2309; Imai (1999) Br J Haematol 106:905-911; Pablos (1999) Am J Pathol 155:1577-1586], such as for example the liver [Shirozu (1995) Genomics 28:495-500; Nagasawa (1996) Nature 382:635-638; Goddard (2001) Transplantation 72:1957-1967]. Previously, the present inventors were able to show that bone marrow homing and repopulation by sorted human CD34⁺/CD38^{low} stem cells transplanted into the tail vein of irradiated immune deficient NOD/SCID and NOD/SCID/B2m null mice, are dependent on SDF-1/CXCR4 interactions [Peled (1999) Science 283:845-848; Kollet (2001) Blood 97:3283-3291].

More recently, the present inventors also established a role for these interactions in G-CSF-induced mobilization of murine and human stem cells [Petit (2002) Nat Immunol 3:687-694].

In view of the ever-expanding use of stem cell therapy, it is highly desirable to further elucidate the mechanism behind stem cell homing and target repopulation in order to improve the efficiency and success rate of cell replacement therapy.

While conceiving the present invention, the present inventors have hypothesized that stress conditions may promote stem cell homing to a target tissue. This hypothesis is strongly supported by prior art studies which illustrated the following:

(i) Stem cells were found to repopulate a damaged murine liver while such finding was not be observed in parabiotic mice [Wagers (2002) Science 297:2256-2259], suggesting that repopulation does not occur under steady state homeostatic conditions in non-irradiated or non-damaged intact livers.

(ii) Although the levels of hematopoietic stem cells that engraft the irradiated liver and develop into hepatocyte-like, albumin producing cells are very low, this process can be amplified by liver injury or viral inflammation. Thus, under

strong selection conditions that exist in fumarylacetoacetate hydrolase (FAH) null mice, which have ongoing severe hepatocyte damage due to deficiency of this enzyme, there is enormous amplification of transplanted, purified murine hematopoietic stem cells that demonstrate hepatic morphology and function, along with improvement of the metabolic disorder [Lagasse (2000) Nat Med 6:1229-1234].

(iii) Liver repopulation by bone marrow (BM) cells from Bcl-2 transgenic mice transplanted into wild-type recipients, followed by repeated rounds of liver injury and regeneration induced by Fas-mediated apoptosis, represents another example of selective amplification of transplanted BM cells following differentiation into hepatocytes [Mallet (2002) Hepatology 35:799-804].

(iv) High levels of bone marrow-derived hepatocytes were reported in a liver transplant recipient in whom the transplanted liver became infected with hepatitis C virus [Theise (2000) Hepatology 32:11-16.].

Altogether these observations demonstrate the potential of hematopoietic stem cells to gain hepatic phenotype can be significantly amplified under stress conditions. However, the mechanisms and factors, which regulate stem cell recruitment to the damaged tissue and induce their desirable phenotype, are currently unknown.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of increasing sensitivity of stem cells to a chemoattractant, the method comprising exposing the stem cells to a matrix metalloprotease or an active portion thereof which is capable of increasing a level of at least one chemoattractant receptor of the stem cells to thereby increase the sensitivity of the stem cells to the chemoattractant.

According to another aspect of the present invention there is provided a method of treating a disorder requiring cell or tissue replacement, the method comprising providing to a subject in need thereof a therapeutically effective amount of stem cells treated with a matrix metalloprotease or an active portion thereof which is capable of increasing a level of at least one chemoattractant receptor of the stem cells, thereby treating the disorder requiring cell or tissue replacement in the subject.

According to yet another aspect of the present invention there is provided a culture medium suitable for increasing the sensitivity of stem cells to a

chemoattractant, the culture medium comprising a matrix metalloprotease or an active portion thereof which is capable of increasing a level of at least one chemoattractant receptor of the stem cells and a buffer solution suitable for stem cell culturing.

According to further features in preferred embodiments of the invention
5 described below, the culture medium further comprises a differentiation inhibiting factor.

According to still further features in the described preferred embodiments the culture medium further comprises serum or serum replacement.

According to still further features in the described preferred embodiments the
10 culture medium further comprises an agent selected from the group consisting of SCF HGF and IL-6.

According to still another aspect of the present invention there is provided a use of a matrix metalloprotease or an active portion thereof for the manufacture of a medicament for increasing homing of stem cells to a target tissue.

According to an additional aspect of the present invention there is provided a
15 method of generating stem cells suitable for transplantation, the method comprising: (a) collecting stem cells; (b) exposing the stem cells to a matrix metalloprotease or an active portion thereof; and (c) isolating stem cells having CXCR4 levels above a predetermined threshold, to thereby generate stem cells suitable for transplantation.

According to still further features in the described preferred embodiments the
20 exposing the stem cells to the matrix metalloprotease or the active portion thereof, is effected by: (i) expressing a polynucleotide encoding the matrix metalloprotease or the active portion thereof in the stem cells; and/or (ii) contacting the stem cells with the matrix metalloprotease or the active portion thereof.

According to still further features in the described preferred embodiments
25 collecting the stem cells is effected by: (i) a stem cell mobilization procedure; and/or (ii) a surgical procedure.

According to still further features in the described preferred embodiments
30 isolating stem cells having CXCR4 levels above the predetermined threshold is effected by FACS.

According to still further features in the described preferred embodiments the method further comprises determining homing capabilities of the stem cells having CXCR4 levels above the predetermined threshold following step (c).

According to yet an additional aspect of the present invention there is provided
5 a nucleic acid construct comprising a first polynucleotide sequence encoding a matrix metalloprotease or an active portion thereof and an inducible cis-acting regulatory element for directing expression of the polynucleotide in cells.

According to still further features in the described preferred embodiments the inducible cis-acting regulatory element is a shear stress activation element.

10 According to still further features in the described preferred embodiments the nucleic acid construct further comprises a second polynucleotide sequence being translationally fused to the first polynucleotide sequence, the second polynucleotide sequence encoding a signal peptide capable of directing secretion of the matrix metalloprotease or the active portion thereof out of the cells.

15 According to still an additional aspect of the present invention there is provided a eukaryotic cell comprising the nucleic acid construct.

According to a further aspect of the present invention there is provided a cell-line comprising stem cells transformed to express an exogenous polynucleotide encoding a matrix metalloprotease.

20 According to yet a further aspect of the present invention there is provided a method of increasing sensitivity of stem cells to a chemoattractant, the method comprising, upregulating an expression or activity of at least one endogenous MMP of the stem cells to thereby increase the sensitivity of the stem cells to the chemoattractant.

25 According to still further features in the described preferred embodiments the at least one chemoattractant receptor is CXCR4.

According to still further features in the described preferred embodiments the matrix metalloprotease is selected from the group consisting of MMP-2, MMP-3, MMP-9, MMP-10, MMP-13 and MMP-14.

30 According to still further features in the described preferred embodiments the matrix metalloprotease is selected from the group consisting of MMP-2 and MMP-9.

According to still further features in the described preferred embodiments the stem cells are hematopoietic stem cells.

According to still further features in the described preferred embodiments the hematopoietic stem cells are CD34⁺ hematopoietic stem cells.

According to still further features in the described preferred embodiments the hematopoietic stem cells are CD34⁺/CD38^{low} hematopoietic stem cells.

5 According to still further features in the described preferred embodiments the stem cells are mesenchymal stem cells.

According to yet a further aspect of the present invention there is provided a method of increasing sensitivity of stem cells to a chemoattractant in a subject in need
10 the method comprising, administering said patient with at least one matrix metalloprotease or an active portion thereof.

The present invention successfully addresses the shortcomings of the presently known configurations by providing stem cells, which exhibit increased sensitivity to a
15 chemoattractant and methods of generating and using the same.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those
20 described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

25 BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and
30 are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the

description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1a-c shows graphs depicting SDF-1/CXCR4-dependent homing of
5 human CD34⁺ cells to different target tissues of NOD/SCID mice. Figure 1a is a histogram showing inhibition of homing of human CB or MPB enriched CD34⁺ cells to the murine BM, spleen and liver by neutralizing CXCR4 antibodies. Data presents inhibition as percentage of control. $P \leq 0.008$, comparing anti CXCR4 treated samples to control counterparts. Figure 1b shows a representative homing experiment
10 showing human CD34⁺/CD38^{-low} homing cells (gated) in the liver of mice transplanted with non-treated cells (upper panel), CXCR4 neutralized cells (middle panel) and non-injected mouse which served as a negative control (lower panel). Figure 1c shows a four hour homing experiment of CXCR4-neutralized or non-treated CD34⁺ cells to the liver of non-irradiated mice. Human SDF-1 was injected to the
15 liver parenchyma as indicated. Cells were collected from the injected lobe to determine the homing of human CD34⁺ cells.

FIG. 2a shows a graph depicting a four hour homing assay of human enriched CD34⁺ cells to the liver of non-irradiated NOD/SCID mice injected with 15 μ l CCl₄ 24 hours prior to the assay.

20 FIG. 2b shows a histogram depicting the levels of human progenitors in mice six weeks following transplantation as determined using peripheral blood mononuclear cells (MNC) of chimeric mice in semisolid cultures one day after a single injection of 10 μ l CCl₄. Data summarize three independent experiments.

FIG. 2c shows a histogram depicting the human CXCR4 staining of peripheral
25 blood MNC from non-treated or CCl₄ injected chimeric mice of Figure 2b.

FIG. 2d shows a photomicrograph depicting a zymography assay showing increased MMP-2/9 activity in the liver of CCl₄ injected mice. Control Blood samples (lanes 1, 2); Blood samples obtained one day following injection of 15 μ l CCl₄ (lane 3); Blood samples obtained two days following injection of 30 μ l CCl₄ (lane 4); Blood
30 samples obtained two days following injection of 15 μ l CCl₄ (lane 5); Conditioned medium enriched with MMP2/9 from HT1080 human cell line (lane 6).

FIG. 2e shows a graph depicting upregulation of CXCR4 expression following treatment with MMP2/9 as determined by FACS analysis. CB CD34⁺ cells were

incubated for 5 hours with RPMI growth medium and HT1080 conditioned medium. Cells were stained with isotype control antibody as a negative control (Isot), or with CXCR4 antibody. Representative data of 3 experiments is shown.

FIG. 2f shows a histogram depicting migration of CB and MPB CD34⁺ cells
5 towards SDF-1 as determined using a transwell system. RPMI (Ctrl), or conditioned medium of the cell line HT1080, enriched with secreted MMP-2/9 (Ginestra 1997. J Biol Chem 272:17216-17222, were added to the upper transwells together with CD34⁺ cells. Cells were incubated with a specific MMP-2/9 inhibitor III (100 μ M, CalBiochem, 30 min.) prior to migration. When added together, HT1080 conditioned
10 medium and MMP2/9 inhibitor were pre-incubated together (30 min.), prior to addition to the cells in the upper transwell. Data represent fold-increased migration compared to control cells.

FIG. 3 shows that MMP-9/2 are involved in homing of MBP CD34⁺ cells to the
15 spleen. MBP CD34⁺ cells were pre-treated for 2 hours with an MMP-9/2 inhibitor and injected into sublethally irradiated NOD/SCID mice (0.5 x10⁵ cells/mouse). Mice were sacrificed 16 hours later and analysed for the presence of human cells/1.5 x 10⁶ acquired cells.

FIG. 4 shows that MMP-9/2 are involved in the SDF-1 mediated in vitro migration of
20 G2 cells. 1x10⁵ G2 cells were either pre-incubated with the MMP-9/2 inhibitor and/or the HT1080 cell line and assayed in a transwell migration assay to 10ng/ml SDF-1.

FIG. 5 shows that purified MMP-2/MMP-9 are involved in in vitro migration of
CD34⁺ cells. 1x10⁵ untreated CB CD34⁺ were assayed in a transwell with purified
25 recombinant MMP-2 or MMP-9 in the presence or absence of MMP-2/MMP-9 inhibitor. Fold increase in migration compares SDF-1 (10 ng/ml) mediated migration of untreated cells in the presence of MMP-9 or MMP

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to stem cells which exhibit increased sensitivity to a chemoattractant and to methods of generating and using the same. Specifically, the present invention allows to treat disorders requiring cell or tissue replacement such as for example to treat chronic or acute liver damage.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

The use of cellular therapy is growing rapidly, and is gradually becoming an important therapeutic modality in treatment of various disorders. Hematopoietic stem cell (HSC) (e.g., from the bone marrow, umbilical cord blood or mobilized peripheral blood) transplantation is one example of a routinely practiced, insurance-reimbursed cellular therapy. However, many other cellular therapies are being developed as well, including immunotherapy for cancer and infectious diseases, chondrocyte therapy for cartilage defects, neuronal cell therapy for neurodegenerative diseases, and stem cell therapy for numerous applications [Forbes (2002) Clinical Science 103:355-369].

One of the problems associated with stem cell therapy is the difficulty of achieving long-term successful engraftment of cells at the target tissue. Currently, patients which were successfully transplanted exhibit very low levels of stem cells and immature progenitors which generate cells with the desired phenotype.

Thus, the success of stem cell transplantation depends on the ability of intravenously infused stem cells to lodge in the target tissue (e.g., bone marrow), a process referred to as homing. It is hypothesized that homing is a multistep process, consisting of adhesion of the stem cells to endothelial cells of the marrow sinusoids, followed by transendothelial migration directed by chemoattractants, and finally anchoring within the extravascular bone marrow spaces where proliferation and differentiation will occur.

Studies have shown that numerous factors are involved in the homing process including, adhesion molecules, cytokines and growth factors. In 1997 studies uncovered that migration of CD34⁺ cells was governed by the chemoattractant, SDF-1. Subsequent studies have shown that SDF-1 activates integrins on HSCs and induces trans-endothelial migration of HSCs in vitro. The receptor for SDF-1 is a G-protein coupled receptor, termed CXCR-4. In SDF-1 or CXCR-4 knock-out mice hematopoietic precursors do not shift to the bone marrow during fetal development suggesting that SDF-1/CXCR-4 interactions play an important role in stem cell migration [for review see Voermans (2001) J. Hematother. Stem Cell Res. 10:725-738, Lapidot (2002) Leukemia 16:1992-2003].

Despite preliminary understanding of the homing process, information about regulation of migration of stem cells is still incomplete and scattered. It is well appreciated that improving the efficacy of stem cell transplantation may be achieved by modulating the ability of stem cells to home to the target tissue.

While reducing the present invention to practice the present inventors have uncovered that matrix metalloprotease activity upregulates CXCR4 expression in hematopoietic stem cells, thereby promoting SDF-1/CXCR4 mediated stem cell homing to damaged organ tissue.

The inventors uncovered that MMP-2/9 is also involved in homing of precursor cells to spleen and bone marrow and in repopulation of such organs also in the absence of inflammation.

In addition the inventors demonstrated that MMP-2/9 action is involved also in the migration of leukemic cells such as pre BLL cell G2.

As illustrated hereinunder and in the Examples section which follows, the present inventors illustrate that hepatic injury upregulates matrix metalloprotease (MMP) activity in the liver, leading to increased CXCR4 expression and SDF-1 mediated homing of hematopoietic progenitor cells to the damaged liver. Furthermore, treatment of CD34⁺ progenitor cells with secreted MMPs upregulates expression of CXCR4 and stem cell migration in-vitro, while addition of an MMP inhibitor completely blocks migration, substantiating the role of MMP in stem cell homing.

Although matrix metalloprotease activity (i.e., MMP-2, 3, 9, 10, 13 and 14) has been previously shown to be upregulated following liver injury [Knittel (2000)

Histochem Cell Biol 113:443-453], the present inventors are the first to show that this upregulation in MMP activity leads to upregulation in CXCR4 expression and to an accelerated homing of cells expressing the same, such as HSCs, in contrary to Knittel's proposed role in ECM remodeling and motility of Hepatic Stellate cells referred to as HSCs.

In addition, although proteolytic enzymes such as elastase, cathepsin-G, MMP-2 and MMP-9 were found to inactivate SDF-1 by cleaving a few amino acids at the N-terminus portion of this chemokine, to thereby create a chemokine that is devoid of chemotaxis [Delgado (2001) Eur. J. Immunol. 31:699; McQuibban (2001) J. Biol. Chem. 276:43503], these events are implicated in stem cell mobilization rather than homing, two mirror image processes utilizing similar mechanisms.

The present findings enable the generation of stem cells, which can be efficiently recruited to a target tissue and as such can be used in numerous clinical applications, such as in repair of liver injury and in liver or bone marrow transplantation.

Thus, according to one aspect of the present invention there is provided a method of increasing sensitivity of stem cells to a chemoattractant.

Also, according to another aspect of the present invention, there is provided a method for inhibiting migration of leukemic cells such as preBLL cells, by using a MMP-9/2 inhibitor.

As used herein, the phrase "stem cells" refers to cells, which are capable of differentiating into other cell types having a particular, specialized function (i.e., "fully differentiated" cells).

The method according to this aspect of the present invention includes exposing the stem cells to a matrix metalloprotease or an active portion thereof which is capable of increasing the level of at least one chemoattractant receptor of the stem cells to thereby increase the sensitivity of the stem cells to the chemoattractant.

Alternatively, increasing sensitivity of stem cells to a chemoattractant can also be effected by upregulating expression or activity of at least one endogenous MMP of the stem cells.

As is further described herein under, exposing the stem cells to a matrix metalloprotease or an active portion thereof can be effected by either contacting the cells with the protein or active portion thereof, or by expressing the protein or active

portion thereof within these cells or in non-stem cells cultured therewith (e.g., fibroblasts used as a feeder layer).

As is clearly demonstrated in the Examples section which follows, exposure of stem cells to MMP substantially increased their ability to home into an injured tissue.

5 Non-limiting examples of stem cells, which can be used according to this aspect of the present invention, are hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) obtained from bone marrow tissue of an individual at any age or from cord blood of a newborn individual, embryonic stem (ES) cells obtained from the embryonic tissue formed after gestation (e.g., blastocyst), or embryonic germ (EG)
10 cells obtained from the genital tissue of a fetus any time during gestation, preferably before 10 weeks of gestation. Further description of stem cells, which can be used according to this aspect of the present invention is summarized hereinbelow.

HSCs - Hematopoietic stem cells (HSCs) are the formative pluripotential blast cells found inter alia in fetal liver, umbilical cord blood, bone marrow and peripheral
15 blood which are capable of differentiating into any of the specific types of hematopoietic or blood cells, such as erythrocytes, lymphocytes, macrophages and megakaryocytes. Typically, within the bone marrow, HSCs reside in niches that support all the requisite factors and adhesive properties to maintain their ability and produce an appropriate balanced output of mature progeny over the life time of the
20 organism [Whetton (1999) Trends Cell Biol 9:233-238; Weissman (2000) Cell 100:157-168; Jankowska-Wieczorek (2001) Stem Cells 19:99-107; Chan (2001) Br. J. Haematol. 112:541-557].

HSCs according to this aspect of the present invention are preferably CD34⁺ cells and more preferably CD34⁺/CD38^{low} cells, which are a more primitive stem cell
25 population and are therefore less lineage-restricted, and were shown to be the major long-term BM repopulating cells.

MSCs - Mesenchymal stem cells are the formative pluripotential blast cells found inter alia in bone marrow, blood, dermis and periosteum that are capable of differentiating into more than one specific type of mesenchymal or connective tissue
30 (i.e. the tissues of the body that support the specialized elements; e.g. adipose, osseous, stroma, cartilaginous, elastic and fibrous connective tissues) depending upon various influences from bioactive factors, such as cytokines.

Approximately, 30 % of human marrow aspirate cells adhering to plastic are

considered as MSCs. These cells can be expanded in vitro and then induced to differentiate. The fact that adult MSCs can be expanded in vitro and stimulated to form bone, cartilage, tendon, muscle or fat cells render them attractive for tissue engineering and gene therapy strategies. In vivo assays have been developed to assay
5 MSC function. MSCs injected into the circulation can integrate into a number of tissues described hereinabove. Specifically, skeletal and cardiac muscle can be induced by exposure to 5-azacytidine and neuronal differentiation of rat and human MSCs in culture can be induced by exposure to β -mercaptoethanol, DMSO or butylated hydroxyanisole [Tomita (1999) 100:11247-11256; Woodbury (2000) J.
10 Neurosci. Res. 61:364-370]. Furthermore, MSC-derived cells are seen to integrate deep into brain after peripheral injection as well as after direct injection of human MSCs into rat brain; they migrate along pathways used during migration of neural stem cells developmentally, become distributed widely and start lose markers of HSC specialization [Azizi (1998) Proc. Natl. Acad. Sci. USA 95:3908-3913]. Methods for
15 promoting mesenchymal stem and lineage-specific cell proliferation are disclosed in U.S. Pat. No. 6,248,587.

Epitopes on the surface of the human mesenchymal stem cells (hMSCs) such as SH2, SH3 and SH4 described in U.S. Pat. No. 5,486,359 can be used as reagents to screen and capture mesenchymal stem cell population from a heterogeneous cell
20 population, such as exists, for example, in bone marrow. Precursor mesenchymal stem cells which are positive for CD45 are preferably used according to this aspect of the present invention, since these precursor mesenchymal stem cells can differentiate into the various mesenchymal lineages.

Preferred stem cells according to this aspect of the present invention are human
25 stem cells.

Table 1, below provides examples of adult stem cells, which can be used to obtain the indicated phenotype in a target tissue of interest, according to this aspect of the present invention.

Table 1

<i>Stem cell</i>	<i>Differentiated phenotype</i>	<i>Target tissue</i>	<i>Reference</i>
Bone marrow	Oval cells, Hepatocytes	Liver	Petersen (1999) Science 284:1168-1170
KTLS cells	Hepatocytes	Liver	Lagasse (2000) Nat. Med. 6:1229-1234
Bone marrow	Hepatocytes	Liver	Alison (2000) Nature 406:257; Thiese (2000) Hepatology 32:11-16
Pacreatic exocrine cells	Hepatocytes	Liver	Shen (2000) Nat. Cell Biol. 2:879-887
Pancreas	Hepatocytes	Liver	Wang (2001) Am. J. Pathol. 158:571-579
Bone marrow	Endothelium	Liver	Gao (2001) Lancet 357:932-933
Bone marrow	Tubular epithelium, glomeruli	Kidney	Poulsom (2001) J. Pathol. 195:229-235
Bone marrow	Endothelium	Kidney	Lagaaij (2001) Lancet 357:33-37
Extra renal	Endothelium	Kidney	Williams (1969) Surg. Forum 20:293-294
Bone marrow	Myocardium	Heart	Orlic (2001) Nature 410:701-704
Bone marrow	Cardiomyocytes and Endothelium	Heart	Jackson (2001) J. Clin Invest. 107:1395-1402
Bone marrow	Type 1 pneumocytes	Lung	Krause (2001) Cell 105:369-377
Neuronal	Multiple hematopoietic lineages	Marrow	Bjornson (1999) Science 283:534-537
Bone marrow	Neurons	CNS	Mezey (2000) Science 290:1779-1782
Bone marrow	Microglia and Astrocytes	CNS	Eglitis (1997) Proc. Natl. Acad. Sci. USA 94:4080-4085

Abbreviations: SP- Side population cells; CNS – central nervous system;

As mentioned hereinabove the stem cells according to this aspect of the present invention are exposed to a matrix metalloprotease (MMP) or an active portion thereof. A matrix metalloprotease (MMP) refers to an enzyme of the MMP family, which are typically known to degrade connective tissues and connective tissue components. MMPs are characterized by a catalytic domain of about 170 amino acids including a zinc binding motif HEXXHXXGXXH and a conserved methionine, which forms a unique "Met-turn" structure. The catalytic domain includes of a five-stranded β -sheet, three α -helices, and bridging loops. MMP-2 and MMP-9 have three repeats of fibronectin-type II domain inserted in the catalytic domain. These repeats interact with collagens and gelatins. The C-terminal hemopexin-like domain including about 210 amino acids has an ellipsoidal disk shape with a four bladed β -propeller structure; each

blade consists of four antiparallel β -strands and an α -helix. The hemopexin domain is an absolute requirement for collagenases to cleave triple helical interstitial collagens, although the catalytic domains alone retain proteolytic activity toward other substrates. The function of the proline-rich linker peptide that connects the catalytic and the hemopexin domains is not known, although its interaction with triple helical collagen is hypothesized based on molecular modeling. MMP-23 has cysteine-rich, proline-rich, and IL-1 receptor-like regions instead of the hemopexin domain. A transmembrane domain is found in the MT-MMPs, which anchors those enzymes to the cell surface. The active portion of the MMP according to this aspect of the present invention preferably refers to the minimal MMP sequence, which is sufficient to increase the sensitivity of the stem cells of the present invention to the chemoattractant. As used herein an active portion of MPP, refers also to a mutein, fusion protein, functional derivative, fragment, circularly permuted MPP and/or salt thereof. To determine the active portion of MMP according to the invention, stem cells can be contacted with an MMP segment and response of the cells thereto can be monitored molecularly, biochemically or functionally (e.g., motility, homing, migration assays) using methods, which are well known to those of skill in the art and further described hereinbelow. Table 2 below, lists a number of vertebrate MMPs, which can be used to increase expression of the chemmoattractant receptor according to this aspect of the present invention.

Table 2

Protein	MMP
Collagenase 1	MMP-1
Gelatinase A	MMP-2
Stromelysin 1	MMP-3
Matrilysin	MMP-7
Collagenase 2	MMP-8
Gelatinase B	MMP-9
Stromelysin 2	MMP-10
Stromelysin 3	MMP-11
Macrophage elastase	MMP-12
Collagenase 3	MMP-13
MT1-MMP	MMP-14
MT2-MMP	MMP-15
MT3-MMP	MMP-16
MT4-MMP	MMP-17
(No trivial name)	MMP-19
Enamelysin	MMP-20
XMMP	MMP-21
CMMP	MMP-22
(No trivial name)	MMP-23

The choice of MMP utilized according to this aspect of the present invention depends on the receptor activated. A number of chemotactic cell receptors are known to participate in transendothelial migration of stem cells. Many of these receptors belong to the family of G protein-coupled seven-transmembrane receptors (7-TMR).
5 Signaling via G proteins, particularly Gi proteins, results in a chemotactic response of the cells towards a gradient of the corresponding ligand [Voermans (2001) J. Hematother. Stem Cell Res. 10:725-738]. Recent studies have provided evidence for expression of several 7-TMR on immature hematopoietic progenitor cells, which potentially mediate chemotactic effects: chemokine receptors (e.g., CXCR4, receptor
10 for stromal cell-derived factor-1), receptors for lipid mediators (e.g., the cysteinyl leukotriene receptor cysLT1 and the peripheral cannabinoid receptor cb2), and receptors for neuroendocrine hormones (e.g., the somatostatin receptor sst2). From these studies it can be concluded that migration of hematopoietic progenitor and stem cells is controlled by a variety of chemotactic factors rather than by a single
15 chemokine (e.g., SDF-1).

Since a number chemoattractant receptors expressed by stem cells have been characterized, the effect of various types of MMPs on expression of these receptors in stem cells can be measured and assessed. Thus, the effect of any MMP or an active portion thereof on chemotactic receptor expression can be determined using
20 biochemical or preferably functional assays, which are well known in the art, several of which are described in detail hereinbelow.

Preferably, the MMP utilized by the method of the present invention is MMP2 and/or MMP9. As is shown in the Examples section which follows, exposure of stem cells to either of these MMPs resulted in upregulation of CXCR4, the G-protein
25 coupled receptor of SDF-1.

As mentioned hereinabove, exposing the stem cells to an MMP or an active portion thereof can be effected by contacting the stem cells with the protein or by expressing the protein within the stem cells.

Contacting stem cells with an MMP or active portion thereof is preferably
30 effected ex-vivo, using harvested cells, although the present invention also contemplates mobilization of stem cells from tissue into circulation and exposure of circulating stem cells to the MMP.

The invention relates to MMP and to its salts, functional derivatives, precursors and active fractions as well as its active mutants, i.e. other proteins or polypeptides wherein one or more amino acids of the structure are eliminated or substituted by other amino acids or one or more amino acids were added to that sequence in order to obtain polypeptides or proteins having the same activity of the MMP and comprises also the corresponding "fusion proteins" i.e. polypeptides comprising the MMP or a mutation thereof fused with another protein. The MMP can therefore be fused with another protein such as, for example, an immunoglobulin.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the MMP protein of the invention or muteins thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulphuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Of course, any such salts must have substantially similar activity to the MMP protein of the invention or its muteins.

The definition "functional derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the terminal N- or C- groups according to known methods and are comprised in the invention when they are pharmaceutically acceptable i.e. when they do not destroy the protein activity or do not impart toxicity to the pharmaceutical compositions containing them. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups.

"Fragment" of the protein the present invention refers to any fragment or precursor of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates, or aggregates of the polypeptide molecule when such fragments or precursors show the same activity of the MMP as medicament.

The term "circularly permuted" as used herein refers to a linear molecule in which the termini have been joined together, either directly or through a linker, to produce a circular molecule, and then the circular molecule is opened at another location to produce a new linear molecule with termini different from the termini in the original molecule. Circular permutations include those molecules whose structure is equivalent to a molecule that has been circularized and then opened. Thus, a circularly permuted molecule may be synthesized de novo as a linear molecule and never go through a circularization and opening step. The particular circular permutation of a molecule is designated by brackets containing the amino acid residues between which the peptide bond is eliminated. Circularly permuted molecules, which may include DNA, RNA and protein, are single-chain molecules which have their normal termini fused, often with a linker, and contain new termini at another position. See Goldenberg, et al. *J. Mol. Biol.*, 165: 407-413 (1983) and Pan et al. *Gene* 125: 111-114 (1993), both incorporated by reference herein. Circular permutation is functionally equivalent to taking a straight-chain molecule, fusing the ends to form a circular molecule, and then cutting the circular molecule at a different location to form a new straight chain molecule with different termini. Circular permutation thus has the effect of essentially preserving the sequence and identity of the amino acids of a protein while generating new termini at different locations.

20

The terms "polypeptide and protein" in the present specification are interchangeable.

The present invention also concerns muteins of the above MMP protein of the invention, which muteins retain essentially the same biological activity of the MMP protein having essentially only the naturally occurring sequences of the MMP. Such "muteins" may be ones in which up to about 20 and 10 amino acid residues may be deleted, added or substituted by others in the MMP protein respectively, such that modifications of this kind do not substantially change the biological activity of the protein mutein with respect to the protein itself.

These muteins are prepared by known synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable thereof.

Any such mutein preferably has a sequence of amino acids sufficiently duplicative of that of the basic the MMP such as to have substantially similar activity

thereto. Thus, it can be determined whether any given mutein has substantially the same activity as the basic protein of the invention by means of routine experimentation comprising subjecting such a mutein to the biological activity tests set forth in Examples below.

5 Muteins of the MMP protein which can be used in accordance with the present invention, or nucleic acid coding thereof, include a finite set of substantially the MMP corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed
10 description of protein chemistry and structure, see Schulz, G.E. et al., Principles of Protein Structure, Springer-Verlag, New York, 1978; and Creighton, T.E., Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. For a presentation of nucleotide sequence substitutions, such as codon preferences, see. See Ausubel et al., Current Protocols in
15 Molecular Biology, Greene Publications and Wiley Interscience, New York, NY, 1987-1995; Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

Preferred changes for muteins in accordance with the present invention are what are known as "conservative" substitutions. Conservative amino acid substitutions
20 of those in the protein having essentially the naturally-occurring MMP sequences, may include synonymous amino acids within a group, which have sufficiently similar physicochemical properties that substitution between members of the group will preserve the biological function of the molecule, see Grantham, Science, Vol. 185, pp. 862-864 (1974). It is clear that insertions and deletions of amino acids may also be
25 made in the above-defined sequence without altering its function, particularly if the insertions or deletions only involve a few amino acids, e.g., under 50, and preferably under 20 MMP and do not remove or displace amino acids which are critical to a functional conformation, e.g., cysteine residues, Anfinsen, "Principles That Govern The Folding of Protein Chains", Science, Vol. 181, pp. 223-230 (1973). Muteins
30 produced by such deletions and/or insertions come within the purview of the present invention.

Preferably, the synonymous amino acid groups are those defined in Table A. More preferably, the synonymous amino acid groups are those defined in Table B; and most preferably the synonymous amino acid groups are those defined in Table C.

5

TABLE A Preferred Groups of Synonymous Amino Acids

	Amino Acid	Synonymous Group
10	Ser	Ser, Thr, Gly, Asn
	Arg	Arg, Gln, Lys, Glu, His
	Leu	Ile, Phe, Tyr, Met, Val, Leu
	Pro	Gly, Ala, Thr, Pro
	Thr	Pro, Ser, Ala, Gly, His, Gln, Thr
15	Ala	Gly, Thr, Pro, Ala
	Val	Met, Tyr, Phe, Ile, Leu, Val
	Gly	Ala, Thr, Pro, Ser, Gly
	Ile	Met, Tyr, Phe, Val, Leu, Ile
	Phe	Trp, Met, Tyr, Ile, Val, Leu, Phe
20	Tyr	Trp, Met, Phe, Ile, Val, Leu, Tyr
	Cys	Ser, Thr, Cys
	His	Glu, Lys, Gln, Thr, Arg, His
	Gln	Glu, Lys, Asn, His, Thr, Arg, Gln
	Asn	Gln, Asp, Ser, Asn
25	Lys	Glu, Gln, His, Arg, Lys
	Asp	Glu, Asn, Asp
	Glu	Asp, Lys, Asn, Gln, His, Arg, Glu
	Met	Phe, Ile, Val, Leu, Met
	Trp	Trp

30

TABLE B More Preferred Groups of Synonymous Amino Acids

	Amino Acid	Synonymous Group
5	Sers	Sers
	Arc	His, Lys, Arg
	Leu	Ile, Phe, Met, Leu
	Pro	Ala, Pro
	Thr	Thr
10	Ala	Pro, Ala
	Val	Met, Ile, Val
	Gly	Gly
	Ilea	Ile, Met, Phe, Val, Leu
	Phe	Met, Tyr, Ile, Leu, Phe
15	Try	Phi, Try
	Cys	Ser, Cys
	His	Arg, Gln, His
	Gln	Glu, His, Gln
	Asn	Asp, Asn
20	Lys	Arg, Lys
	Asp	Asn, Asp
	Glu	FLN, Glu
	Met	Phe, Ile, Val, Leu, Met
	Trp	Trp

25

30

TABLE C Most Preferred Groups of Synonymous Amino Acids

Amino Acid		Synonymous Group
	Sers	Sers
	Arc	Arc
5	Leu	Ile, Met, Leu
	Pro	Pro
	Thr	Thar
	Alan	Alan
	Val	Val
10	Gly	Gly
	Ilea	Ile, Met, Leu
	Phi	Phi
	Try	Tyr
	Cys	Ser, Cys
15	His	His
	Gln	Gln
	Asn	Asn
	Lys	Lys
	Asp	Asp
20	Glu	Glu
	Met	Ile, Leu, Met
	Trp	Trp

25 Examples of production of amino acid substitutions in proteins which can be used for obtaining muteins of the protein for use in the present invention include any known method steps, such as presented in US patents RE 33,653, 4,959,314, 4,588,585 and 4,737,462, to Mark et al; 5,116,943 to Koths et al., 4,965,195 to Namen et al; 4,879,111 to Chong et al; and 5,017,691 to Lee et al; and lysine substituted proteins
 30 presented in US patent No. 4,904,584 (Straw et al).

In another preferred embodiment of the present invention, any mutein of the MMP protein for use in the present invention has an amino acid sequence essentially corresponding to that of the above noted MMP protein of the invention. The term "essentially corresponding to" is intended to comprehend muteins with minor changes to the sequence of the basic protein which do not affect the basic characteristics thereof, particularly insofar as its ability to the MMP is concerned. The type of changes which are generally considered to fall within the "essentially corresponding to" language are those which would result from conventional mutagenesis techniques of the DNA encoding the MMP protein of the invention, resulting in a few minor modifications, and screening for the desired activity for example increasing the sensitivity of stem cells to a chemoattractant.

The present invention also encompasses MMP variants. A preferred MMP variant are the ones having at least 80% amino acid identity, a more preferred the MMP variant is one having at least 90% identity and a most preferred variant is one having at least 95% identity to MMP amino acid sequence.

The term "sequence identity" as used herein means that the amino acid sequences are compared by alignment according to Hanks and Quinn (1991) with a refinement of low homology regions using the Clustal-X program, which is the Windows interface for the ClustalW multiple sequence alignment program (Thompson et al., 1994). The Clustal-X program is available over the internet at <ftp://ftp-igbmc.u-strasbg.fr/pub/clustalx/>. Of course, it should be understood that if this link becomes inactive, those of ordinary skill in the art could find versions of this program at other links using standard internet search techniques without undue experimentation. Unless otherwise specified, the most recent version of any program referred herein, as of the effective filing date of the present application, is the one, which is used in order to practice the present invention.

Another method for determining "sequence identity" is the following. The sequences are aligned using Version 9 of the Genetic Computing Group's GDAP (global alignment program), using the default (BLOSUM62) matrix (values -4 to +11) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (per each additional consecutive null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the claimed sequence.

Mutins in accordance with the present invention include those encoded by a nucleic acid, such as DNA or RNA, which hybridizes to DNA or RNA under stringent conditions and which encodes a the MMP protein in accordance with the present invention, comprising essentially all of the naturally-occurring sequences encoding the
5 MMP and sequences which may differ in its nucleotide sequence from the naturally-derived nucleotide sequence by virtue of the degeneracy of the genetic code, i.e., a somewhat different nucleic acid sequence may still code for the same amino acid sequence, due to this degeneracy.

The term "hybridization" as used herein shall include any process by which a strand of
10 nucleic acid joins with complementary strand through a base pairing (Coombs J, 1994, Dictionary of Biotechnology, stokton Press, New York NY). "Amplification" is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art (Dieffenbach and Dveksler, 1995, PCR Primer, a Laboratory Manual, Cold Spring
15 Harbor Press, Plainview NY).

"Stringency" typically occurs in a range from about $T_m - 5^\circ\text{C}$ (5°C below the melting temperature of the probe) to about 20°C to 25°C below T_m .

The term "stringent conditions" refers to hybridization and subsequent washing conditions, which those of ordinary skill in the art conventionally refer to as
20 "stringent". See Ausubel et al., Current Protocols in Molecular Biology, Greene Publications and Wiley Interscience, New York, NY, 1987-1995; Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

As used herein, stringency conditions are a function of the temperature used in the
25 hybridization experiment, the molarity of the monovalent cations and the percentage of formamide in the hybridization solution. To determine the degree of stringency involved with any given set of conditions, one first uses the equation of Meinkoth et al. (1984) for determining the stability of hybrids of 100% identity expressed as melting temperature T_m of the DNA-DNA hybrid:

$$30 \quad T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$$

where M is the molarity of monovalent cations, %GC is the percentage of G and C nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. For each 1 C that the T_m is

reduced from that calculated for a 100% identity hybrid, the amount of mismatch permitted is increased by about 1%. Thus, if the T_m used for any given hybridization experiment at the specified salt and formamide concentrations is 10 C below the T_m calculated for a 100% hybrid according to the equation of Meinkoth, hybridization will occur even if there is up to about 10% mismatch.

As used herein, "highly stringent conditions" are those which provide a T_m which is not more than 10 C below the T_m that would exist for a perfect duplex with the target sequence, either as calculated by the above formula or as actually measured. "Moderately stringent conditions" are those, which provide a T_m , which is not more than 20 C below the T_m that would exist for a perfect duplex with the target sequence, either as calculated by the above formula or as actually measured. Without limitation, examples of highly stringent (5-10 C below the calculated or measured T_m of the hybrid) and moderately stringent (15-20 C below the calculated or measured T_m of the hybrid) conditions use a wash solution of 2 X SSC (standard saline citrate) and 0.5% SDS (sodium dodecyl sulfate) at the appropriate temperature below the calculated T_m of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those, which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE (standard saline-phosphate-EDTA), 5 X Denhardt's reagent, 0.5% SDS, 100 μ g/ml denatured, fragmented salmon sperm DNA at a temperature approximately 20 to 25 C below the T_m . If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC (Ausubel, 1987, 1999).

Adult stem cells can be obtained using a surgical procedure such as bone marrow aspiration or can be harvested using commercial systems such as those available from Nexell Therapeutics Inc. Irvine, CA, USA.

Stem cells utilized by the present invention are preferably collected (i.e., harvested) using a stem cell mobilization procedure, which utilizes chemotherapy or cytokine stimulation to release of HSCs into circulation of subjects. Stem cells are preferably retrieved using this procedure since mobilization is known to yield more HSCs and progenitor cells than bone marrow surgery.

Stem cell mobilization can be induced by a number of molecules. Examples include but are not limited to cytokines such as, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-7, IL-3, IL-12, stem cell factor (SCF), and flt-3 ligand; chemokines like IL-8, Mip-1 α , Gro β , or SDF-1; and the chemotherapeutic agents cyclophosphamide (Cy) and paclitaxel. It will be appreciated that these molecules differ in kinetics and efficacy, however, according to presently known embodiments G-CSF is preferably used alone or in combination such as with cyclophosphamide to mobilize the stem cells. Typically, G-CSF is administered daily at a dose of 5-10 μ g/kg for 5-10 days. Methods of mobilizing stem cells are disclosed in U.S. Pat. Nos. 6,447,766 and 6,162,427.

Human embryonic stem cells can be isolated from human blastocysts. Human blastocysts are typically obtained from human *in vivo* preimplantation embryos or from *in vitro* fertilized (IVF) embryos. Alternatively, a single cell human embryo can be expanded to the blastocyst stage. For the isolation of human ES cells the zona pellucida is removed from the blastocyst and the inner cell mass (ICM) is isolated by immunosurgery, in which the trophectoderm cells are lysed and removed from the intact ICM by gentle pipetting. The ICM is then plated in a tissue culture flask containing the appropriate medium which enables its outgrowth. Following 9 to 15 days, the ICM derived outgrowth is dissociated into clumps either by a mechanical dissociation or by an enzymatic degradation and the cells are then re-plated on a fresh tissue culture medium. Colonies demonstrating undifferentiated morphology are individually selected by micropipette, mechanically dissociated into clumps, and re-plated. Resulting ES cells are then routinely split every 1-2 weeks. For further details on methods of preparation human ES cells see Thomson et al., [U.S. Pat. No. 5,843,780; Science 282: 1145, 1998; Curr. Top. Dev. Biol. 38: 133, 1998; Proc. Natl. Acad. Sci. USA 92: 7844, 1995]; Bongso et al., [Hum Reprod 4: 706, 1989]; Gardner et al., [Fertil. Steril. 69: 84, 1998].

It will be appreciated that commercially available stem cells can be also be used according to this aspect of the present invention. Human ES cells can be purchased from the NIH human embryonic stem cells registry (<<http://escr.nih.gov>>). Non-limiting examples of commercially available embryonic stem cell lines are BG01, BG02, BG03, BG04, CY12, CY30, CY92, CY10, TE03, TE32.

Human EG cells can be retrieved from the primordial germ cells obtained from human fetuses of about 8-11 weeks of gestation using laboratory techniques known to anyone skilled in the arts. The genital ridges are dissociated and cut into small chunks, which are thereafter disaggregated into cells by mechanical dissociation. The EG cells
5 are then grown in tissue culture flasks with the appropriate medium. The cells are cultured with daily replacement of medium until a cell morphology consistent with EG cells is observed, typically after 7-30 days or 1-4 passages. For additional details on methods of preparing EG cells see Shambloott et al., [Proc. Natl. Acad. Sci. USA 95: 13726, 1998] and U.S. Pat. No. 6,090,622.

10 It will be appreciated that enrichment of stem cell population exhibiting pluripotency may be preferably effected. Thus, for example, as outlined hereinabove, CD34⁺ stem cells can be concentrated using affinity columns or FACS as further described hereinunder.

Culturing of stem cells under proliferative conditions may also be effected in
15 cases where stem cell numbers are too low for use in treatment. Culturing of stem cells is described in U.S. Pat. Nos. 6,511,958, 6,436,704, 6,280,718, 6,258,597, 6,184,035, 6,132,708 and 5,837,5739.

Once stem cells are obtained, they are contacted with a soluble matrix metalloprotease or an active portion thereof.

20 Soluble matrix metalloproteases, and in particular, active portions thereof can be biochemically synthesized by using, for example, standard solid phase techniques. These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis. Solid phase peptide synthesis procedures are well known in the art and further described by John Morrow
25 Stewart and Janis Dillaha Young, Solid Phase Peptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

Synthetic peptides can be purified by preparative high performance liquid chromatography [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via amino
30 acid sequencing.

Some soluble matrix metalloproteases can also be obtained from commercial suppliers such as, for example, MegaPharm, Oncogene Research Products, Hod-Hasharon, Israel.

In cases where large amounts of the soluble matrix metalloprotease or the active portion thereof are desired, such polypeptides are preferably generated using recombinant techniques.

To recombinantly synthesize such polypeptides, an expression construct (i.e.,
5 expression vector), which includes a polynucleotide encoding the soluble matrix metalloprotease or the active portion thereof positioned under the transcriptional control of a regulatory element, such as a promoter, is introduced into host cells.

The "transformed" cells are cultured under suitable conditions, which allow the expression of the protein encoded by the polynucleotide.

10 Following a predetermined time period, the expressed protein is recovered from the cell or cell culture, and purification is effected.

A variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the modified polypeptide coding sequence. These include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant
15 bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the desired coding sequence; mammalian expression systems are preferably used to express the soluble matrix metalloprotease or the active portion thereof, since eukaryotic cells enable the generation of post-translational modified proteins. However, bacterial systems are typically used to produce recombinant proteins since
20 they enable a high production volume at low cost. Thus, the host system is selected according to the recombinant protein to be generated and the end use thereof.

In bacterial systems, a number of expression vectors can be advantageously selected depending upon the use intended for the modified polypeptide expressed. For example, when large quantities of conjugates are desired, vectors that direct the
25 expression of high levels of the protein product, possibly as a fusion with a hydrophobic signal sequence, which directs the expressed product into the periplasm of the bacteria or the culture medium where the protein product is readily purified may be desired. Certain fusion protein engineered with a specific cleavage site to aid in recovery of the conjugate may also be desirable. Such vectors adaptable to such
30 manipulation include, but are not limited to, the pET series of E. coli expression vectors [Studier et al. (1990) Methods in Enzymol. 185:60-89].

Other expression systems such as insects and mammalian host cell systems, which are well known in the art can also be used by the present invention (U.S. Pat. No. 6,541,623),

In any case, transformed cells are cultured under effective conditions, which
5 allow for the expression of high amounts of recombinant polypeptide. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce the recombinant modified polypeptide of the present invention. Such a medium typically includes an
10 aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such
15 culturing conditions are within the expertise of one of ordinary skill in the art.

The resultant recombinant proteins of the present invention are preferably secreted into the growth (e.g., fermentation) medium.

Following a predetermined time in culture, recovery of the recombinant protein is effected. The phrase "recovering the recombinant protein" refers to collecting the
20 whole growth medium containing the protein and need not imply additional steps of separation or purification. Proteins of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase
25 chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

Proteins of the present invention are preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein in the diverse applications, described hereinbelow.

30 It will be appreciated that recombinant production of the MMP or the active portion thereof of the present invention can also be effected in-vitro.

The MMP or active portion thereof can be included in a culture medium utilized for culturing or sustaining the harvested stem cells. Such a culture medium

typically includes a buffer solution (i.e., growth medium) suitable for stem cell culturing. The culture medium can also include serum or serum replacement which includes growth factors which support growth and survival of the stem cells. The culture medium can also include an agent such as SDF-1, IL-6, SCF, HGF and the like, which can promote cell growth, survival differentiation and homing. Additionally the growth medium of the present invention may also include differentiation-inhibiting agents such as leukemia inhibitor factor (LIF).

The stem cells of the present invention can also be contacted with MMP expressing and optionally presenting cells (i.e., insoluble-membrane bound MMP). This can be effected by co-culturing the stem cells of the present invention with cells which express a secreted or membrane-bound MMP. For example, fibroblast feeder cells, which are oftentimes-co-cultured with stem cells to support proliferation thereof in a non-differentiated state can express an MMP of interest, thereby performing a dual role i.e., growth support and increase of homing potential of stem cells.

However since the stem cells of the present invention are preferably used for clinical applications, measures are taken to isolate the stem cells from the second MMP-expressing cell population following induction of sufficient level of the at least one chemoattractant receptor of the stem cells. Methods of sorting cell populations are further described hereinbelow.

Alternatively, the stem cells of the present invention can be transformed with an expression construct such as that described above in order to express the matrix metalloprotease or the active portion thereof in the stem cells.

In such cases, the expression construct includes a cis-acting regulatory element active in mammalian cells (examples above), preferably under inducible, growth specific or tissue specific conditions.

Examples of cell type-specific and/or tissue-specific promoters include promoters such as albumin that is liver specific [Pinkert et al., (1987) Genes Dev. 1:268-277], lymphoid specific promoters [Calame et al., (1988) Adv. Immunol. 43:235-275]; in particular promoters of T-cell receptors [Winoto et al., (1989) EMBO J. 8:729-733] and immunoglobulins; [Banerji et al. (1983) Cell 33:729-740], neuron-specific promoters such as the neurofilament promoter [Byrne et al. (1989) Proc. Natl. Acad. Sci. USA 86:5473-5477], pancreas-specific promoters [Edlunch et al. (1985) Science 230:912-916] or mammary gland-specific promoters such as the milk whey

promoter (U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). The nucleic acid construct of the present invention can further include an enhancer, which can be adjacent or distant to the promoter sequence and can function in up regulating the transcription therefrom.

5 Preferably, the inducible cis-acting regulatory element is regulatable by changes in the environment of the stem cells during the homing-implantation process.

During their migration, stem cells are subjected to shear forces generated by movement of the cells within circulating blood; once implanted, stem cells are no longer subjected to such shear forces. Since the MMP need only be active during the
10 homing stage (migration), the use of a cis-acting regulatory element which is active only at the stage of migration is particularly advantageous. One such regulatory element is the shear stress responsive element described by Resnick et al., in PNAS USA 90:4591-4595, 1993.

Genetic modification of mesenchymal stem cells is discussed in U.S. Pat. No.
15 5,591,625. Genetic modification of HSCs is discussed in Zheng 2000 Nat. Biotechnol. 18:176-180 and Lotti 2002 J. Virol. 76(8)3996-4007.

Once exposed to the MMP or active portion thereof, stem cells exhibiting increased expression levels of the chemoattractant receptor and as a result, increased sensitivity to the chemoattractant are preferably identified and isolated. Although such a step
20 enriches for highly chemotactic cells, use of a non-enriched MMP-treated population is also envisaged by the present invention.

Identification and isolation of such cells according to this aspect of the present invention can be effected using a number of cytological, biochemical and molecular methods which are well known in the art.

25 For example, analysis of receptor level can be effected by flow cytometry. This approach employs instrumentation that scans single cells flowing past excitation sources in a liquid medium. The technology can provide rapid, quantitative, multiparameter analyses on single living (or dead) cells based on the measurement of visible and fluorescent light emission. This basic protocol focuses on: measure
30 fluorescence intensity produced by fluorescent-labeled antibodies and ligands that bind specific cell-associated molecules. To isolate cell populations using fluorescence activated cell sorter stem cells of the present invention are contacted with anti CXCR4 commercially available from R&D, 614 McKinley Place NE Minneapolis, MN.

Other cytological or biochemical methods for quantitatively assessing the level of the chemotactic receptor expression include but are not limited to binding analysis using a labeled (e.g., radioactively labeled) chemokine, western blot analysis, cell-surface biotinylation and immunofluorescent staining.

5 It will be appreciated that the receptor expression levels can also be determined at the mRNA level. For example, CXCR4 mRNA may be detected in cells by hybridization to a specific probe. Such probes may be cloned DNAs or fragments thereof, RNA, typically made by in-vitro transcription, or oligonucleotide probes, usually generated by solid phase synthesis. Methods for generating and using probes
10 suitable for specific hybridization are well known and used in the art. Quantification of mRNA levels can be also effected using an amplification reaction [e.g., PCR, "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990)], employing primers, which hybridize specifically to the mRNA of a chemotactic receptor of interest.

15 A variety of controls may be usefully employed to improve accuracy in mRNA detection assays. For instance, samples may be hybridized to an irrelevant probe and treated with RNase A prior to hybridization, to assess false hybridization.

Functional assays can also be used to determine the chemotactic receptor expression. For example, a chemotaxis assay which employs a gradient of the
20 chemotactic agent (e.g., SDF-1) and follows stem cell migration through a membrane towards the chemotactic agent can be utilized to identify and isolate stem cells exhibiting increased chemotaxis. If the cells do not express enough levels of the chemotactic receptor (e.g., CXCR4), then the majority of the cells will remain on the membrane. However, upon increased expression of the chemoattractant receptor of the
25 present invention, cells will migrate through the membrane and settle on the bottom of the well of the chemotaxis plate (see Example 2 of the Examples section).

It will be appreciated that a functional homing assay can also be utilized by the method of the present invention. Such an assay is described in Kollet (2001) Blood 97:3283-3291.

30 Stem cells exhibiting an increased sensitivity to the chemoattractant can be used in a wide range of clinical applications.

Thus, according to another aspect of the present invention there is provided a method of treating a disorder requiring cell or tissue replacement. The method is

effected by providing to a subject in need thereof a therapeutically effective amount of stem cells treated with a matrix metalloprotease or an active portion thereof which is capable of increasing a level of at least one chemoattractant receptor of the stem cells as described hereinabove, to thereby treat the disorder requiring the cell or tissue replacement in the subject.

Disorders requiring cell or tissue replacement include but are not limited to various immunodeficiencies such as in T and/or B lymphocytes, or immune disorders, such as rheumatoid arthritis. Such immunodeficiencies may be the result of viral infections, HTLV I, HTLV II, HTLV III, severe exposure to radiation, cancer therapy or the result of other medical treatment; Hematological deficiencies including but not limited to leukemias, such as acute lymphoblastic leukemia (ALL), acute nonlymphoblastic leukemia (ANLL), acute myelocytic leukemia (AML) or chronic myelocytic leukemia (CML). Other such hematological deficiencies can be, but are not limited to, severe combined immunodeficiency (SCID) syndromes [such as, for example adenosine deaminase (ADA) deficiency and X-linked SCID (XSCID)], osteopetrosis, aplastic anemia, Gaucher's disease, thalassemia and other congenital or genetically-determined hematopoietic abnormalities; Other disorders requiring cell or tissue replacement include those associated with liver failure, pancreatic failure, neurological disorders, those disorders requiring augmented bone formation such as osteoarthritis, osteoporosis, traumatic or pathological conditions involving any of the connective tissues, such as bone defects, connective tissue defects, skeletal defects or cartilage defects.

Preferred individual subjects according to the present invention are mammals such as canines, felines, ovines, porcines, equines, bovines and preferably humans.

The stem cells according to this aspect of the present invention are preferably obtained from the subject to be treated. However stem cells may also be obtained from a syngeneic, allogeneic and less preferably from a xenogeneic donor.

It will be appreciated that when allogeneic or xenogeneic stem cells are used, the recipient subject and/or cells are preferably treated to prevent graft versus host and host versus graft rejections. Immunosuppression protocols are well known in the art and some are disclosed in U.S. Pat. No. 6,447,765.

It will be appreciated that the stem cells of the present invention can be genetically modified to express any therapeutic gene such as an antiviral agent against hepatitis further described in U.S. Pat. No. 5,928,638.

5 The stem cells are transplanted into the recipient subject. This is generally effected using methods well known in the art, and usually involves injecting or introducing the treated stem cells into the subject using clinical tools well known by those skilled in the art (U.S. Pat. No. 6,447,765, 6,383,481, 6,143,292, and 6,326,198).

10 For example, introduction of the stem cells of the present invention can be effected locally or systematically via intravascular administration, including intravenous or intraarterial administration, intraperitoneal administration, and the like. Cells can be injected into a 50 mol Fenwall infusion bag using sterile syringes or other sterile transfer mechanisms. The cells can then be immediately infused via IV administration over a period of time, such as 15 minutes, into a free flow IV line into
15 the patient. In some embodiments, additional reagents such as buffers or salts may be added as well. The composition for administration must be formulated, produced and stored according to standard methods complying with proper sterility and stability.

Stem cell dosages can be determined according to the prescribed use. In general, in the case of parenteral administration, it is customary to administer from
20 about 0.01 to about 5 million cells per kilogram of recipient body weight. The number of cells used will depend on the weight and condition of the recipient, the number of or frequency of administrations, and other variables known to those of skill in the art.

After administering the cells into the subject, the effect of the treatment may be evaluated, if desired, as known in the art. The treatment may be repeated as needed.
25 The invention also provides a pharmaceutical composition comprising a therapeutically effective amount of an MMP or active portion thereof for treating a disorder requiring cell or tissue replacement.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following
30 examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized
5 in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and
10 Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531;
15 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available
20 immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984);
25 "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al.,
30 "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the

convenience of the reader. All the information contained therein is incorporated herein by reference.

5

EXAMPLE 1

SDF-1/CXCR4 interactions mediate homing of human CD34⁺ progenitor cells to the liver in NOD/SCID mice

10 To examine the role of SDF-1 in human stem cells (HSCs) recruitment to the liver, irradiated NOD/SCID mice were transplanted with human CD34⁺ enriched cells from mobilized peripheral blood or cord blood, with and without neutralizing CXCR4 antibodies, and homing thereof was assayed.

Materials and Experimental Procedures

15 *Human cells* – Cord blood (CB) cells and adult mobilized peripheral blood (MPB) cells were obtained after informed consent in accordance with procedures approved by the human ethics committee of the Weizmann Institute. CD34⁺ cell enrichment was effected using magnetic bead separation as previously described [Kollet (2001) Blood 97:3283-3291]. CXCR4 expression was determined by flow
20 cytometry using purified anti human CXCR4 (clone 12G5, R&D, Minneapolis, MN) and secondary F(ab')₂ fragment of goat anti mouse IgG FITC (Jackson, West Grove, PA).

Mice - NOD/SCID mice were bred and housed as previously described [Kollet (2001) Blood 97:3283-3291]. All experiments were approved by the animal care
25 committee of the Weizmann Institute. Mice were sublethally irradiated (i.e., 375 cGy) as indicated, 24 hours prior to transplantation. Non-irradiated mice were used when local hepatic injection of SDF-1 was effected.

CXCR4 neutralization - Human CD34⁺ cells were pre-incubated with anti human CXCR4 neutralizing mAb (10 µg/0.5x10⁶ cells, 12G5, R&D) and were injected
30 (0.5-0.6x10⁶ CD34⁺ cells per mouse) into the tail vein without washing. Mice were killed 4 hours, 16 hours, or 5-6 weeks following cell transplantation as indicated. Single cell suspensions of liver tissues were washed thoroughly with PBS. Homing of human cells was determined as described [Kollet (2001) Blood 97:3283-3291],

acquiring 1.5×10^6 cells/sample.

Results

CXCR4 neutralization significantly inhibited the homing of human CB or MPB CD34⁺ enriched cells to the BM, spleen and liver of NOD/SCID recipients 16 hours post transplantation (Figure 1a). Interestingly, the more primitive, undifferentiated CD34⁺/CD38^{low} cells, highly enriched for human HSC [Peled (1999) Science 283:845-848] and cells with hepatic-like potential [Danet (2002) Proc Natl Acad Sci U S A 99:10441-10445; Wang (2003) Blood (epub ahead of print)] also required SDF-1/CXCR4 interactions for migration thereof to the murine liver (Figure 1b).

Furthermore, following local injection of human SDF-1 into the hepatic parenchyma of non-irradiated NOD/SCID recipients, and intravenous (IV) infusion of enriched human CD34⁺ cells, it was evident that SDF-1 increased the homing of CD34⁺ progenitors, while neutralizing CXCR4 antibodies almost completely abrogated homing thereof (Figure 1c).

Altogether, these findings show that local tissue expression of SDF-1 plays a chemotactic role in the migration of human stem and progenitor cells to the irradiated murine liver.

EXAMPLE 2

20 *Stress-induces CXCR4⁺ hematopoietic progenitors recruitment to an injured liver*

Liver injury has been found to increase the levels of transplanted rodent bone marrow progenitor cells exhibiting an hepatic phenotype in the rat and murine liver [Petersen (1999) Science 284:1168-1170; Theise (2000) Hepatology 31:235-240; Lagasse (2000) Nat Med 6:1229-1234]. Carbon tetrachloride (CCl₄)-induced liver injury one month post transplantation, in combination with hepatic growth factor (HGF) stimulation, significantly increased the levels of hepatic-like differentiation and human albumin production in immune deficient NOD/SCID and NOD/SCID/B2m null mice engrafted with human CD34⁺ and CD34⁺/CD38⁻ progenitors, revealing <1% of human albumin producing cells in the murine liver 2 months post transplantation [Wang (2003) Blood (epub ahead of print)], supported in another report utilizing a different protocol [Kakinuma (2003) Stem Cells 21:217-227].

Experimental procedures

Liver injury - Mice were injected intraperitoneally (IP) with 10, 15 or 30

μ l/mouse of CCl_4 and liver samples were collected within a few hours, or 1-2 days following injection, as indicated. In homing assays, mice were intravenously (IV) transplanted with human MPB CD34^+ cells (0.6×10^6 cells/mouse) 4 hours prior to liver collection. Homing was blocked by preincubation of transplanted cells with 10 μ g of anti CXCR4/mouse or by IP injection of 100 μ g/mouse of a specific MMP2/9 inhibitor III (CalBiochem, catalogue #444251). Human progenitors in the blood circulation of engrafted mice transplanted a month before with human CB MNC (20×10^6 cells/mouse) were quantified by seeding 2×10^5 mononuclear cells/ml in colony forming unit assay as described [Kollet (2001) Blood 97:3283-3291]. CXCR4 expression was determined by flow cytometry.

Results

As shown in Figure 2a, a single injection of CCl_4 rapidly induced homing of enriched human CD34^+ cells to the liver of treated mice in a CXCR4 dependent manner. Interestingly, CCl_4 -mediated liver injury also induced the recruitment of human colony forming progenitors from the bone marrow to the circulation of engrafted NOD/SCID mice (Figure 2b). Unexpectedly, an increased level of CXCR4 expression on human MNC cells was observed in the circulation of CCl_4 treated mice (Figure 2c). In addition, CCl_4 treatment resulted in increased activity of the proteolytic enzyme MMP-2 and emergence of MMP-9 expression in the liver of treated NOD/SCID mice (Figure 2d). These results show a role for metalloproteases in homing of human CD34^+ stem cells to the injured liver.

To further substantiate the role of MMP-2 and MMP-9 in recruitment of hematopoietic progenitors to the injured liver, migration assays were effected in the presence of soluble MMP-2/9.

Supernatants from HT1080 human cell line, which secrete MMP-2 and MMP-9, were found to increase surface CXCR4 expression on enriched human CD34^+ cells (Figure 2e). Moreover, MMP enriched supernatants significantly increased SDF-1 mediated migration of human progenitors in vitro a migration which was further inhibited in the presence of an MMP-2/9 inhibitor (Figure 2f), demonstrating that these proteolytic enzymes directly affect the motility of enriched human CD34^+ progenitors. Similar results were observed using purified MMP-2 or purified MMP-9 instead of HT1080 sup (Figure 5). As described hereinabove, this inhibitor also reduced the migration of human CD34^+ progenitors to the injured liver in-vivo (Figure 2a),

demonstrating a central role for these proteolytic enzymes in SDF-1 mediated recruitment of hematopoietic progenitors to sites of inflammation in the injured liver.

EXAMPLE 3

5 *Involvement of MMP-9/2 in migration and repopulation of precursor cells to the bone marrow and spleen in the absence of inflammation*

The present work was aimed to uncover whether MMP-2/9 is also involved in homing of precursor cells to spleen and bone marrow and in repopulation of such
10 organs also in the absence of inflammation.

Initially, human cord blood CD34+ cells were treated for 2 hours with an MMP-9/2 inhibitor and injected in sublethally irradiated NOD/SCID mice.

CD34+ cells were pretreated for 2 hours with an MMP-9/2 inhibitor and injected into subletally irradiated NOD/SCID mice (1-2x10⁵ cells/mouse). Mice were
15 sacrificed 5 weeks later and murine BM was labeled for the human pan leukocyte marker CD45 and assayed by FACS. 5 weeks later the mice were sacrificed and the number of human cells was measured in the murine BM. Table 1 shows that engraftment to the BM appears to be inhibited in MMP-2/9 inhibitor treated cells when compared to control non-treated cells.

20 **Table 1 Inhibition of MMP-9/2 impairs CB CD34+ repopulation in BM NOD/SCID mice.**

Experiment	Control (% of total cell injected)	Inhibitor (% of total cell injected)
Exp 1 (2 mice per group)	74.76, 30.2	21, 8
Exp 2 (2 mice per group)	33, 32	18,13

25

It was further explored whether MMP-9/2 are also involved in homing of CD34+ cells to the spleen and bone marrow. Thus, mobilized peripheral blood (MPB) CD34+ cells were pre-treated for 2 hours with MMP-2/9 inhibitor and injected into subletally irradiated NOD/SCID mice (0.5x10⁵ cells/mouse). Mice were sacrificed 16
30 hours later and analyzed for the presence of human cells/1.5 x 10⁶ acquired cells.

The results in figure 3 show that MMP-9/2 inhibitor significantly inhibits migration of MPB CD34+ cells to the spleen but not to the bone marrow. Therefore, this result suggests that MMP-9/2 is involved in homing to the spleen.

It was previously shown that incubation of supernatants from HT1080 human cell line, which secrete MMP-2 and MMP-9, were found to increase surface CXCR4 expression on enriched human CD34+ cells (Example 2). Thus, such increase in the surface CXCR4 expression by MMP-2 and MMP-9 may account for the observed induction of homing and repopulation.

Apparently, the results that MMP-9/2 is required for repopulation of the bone marrow (Table 1) but not for the migration to this same organ (Fig. 3) may be due to migration of transplanted cells to the bone marrow prior to repopulation indirectly via the spleen. In this setting, MMP-9/2 may be required for homing to the spleen first and inhibition of the proteases will result in inhibition of homing to the spleen and indirectly to the inhibition of bone marrow repopulation.

15

EXAMPLE 4

Involvement of MMP-9/2 in migration of PreBLL cells

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As mentioned above (Example 2 and 3), MMP-9/2 action is involved in the mechanism governing migration and repopulation of normal hematopoietic precursors cells. Next, it was explored whether such proteases action is involved also in migration of leukemic cells. For this purpose, the effect of MMP-9/2 inhibitor to in-vitro migration of the pre BLL cell G2, which is a lymphoma which arise from precursors of B cells, to SDF-1 was monitored (Fig. 4).

To explore the involvement of MMP-9/2 on the migration of G2, G2 cells (1x10⁵ G2 cells) were either pre-incubated with the MMP-9/2 inhibitor or with the HT1080 cell line supernatant, which secrete MMP-2 and MMP-9, and assayed in a transwell migration assay to SDF-1 (10ng/ml). The results depicted in Figure 4 shows that G2 cells produce MMP-9/2 which is required for migration to SDF-1. The results show that the addition of ectopic MMP9/2 did not enhance further the migration of G2

30

cells to SDF-1. Also the inhibition of migration by MMP-9/2 was not blocked by the ectopic addition of MMP9/2.

The results obtained show that MMP-9/2 is involved in SDF-1 mediated migration, not only in of normal progenitor cells, but also in leukemic cells developing from B
5 cell precursors.

The results also show that the MMP9/2 inhibitor efficiently inhibits the migration of leukemic G2 cells even in the presence of exogenously added MMP9/2.

Although the invention has been described in conjunction with specific
10 embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference
15 into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.